

Subcellular targeting by membrane lipids

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The reversible localization of signaling proteins to both the plasma and the internal membranes of cells is critical for the selective activation of downstream functions and depends on interactions with both proteins and membrane lipids. New structural and biochemical analyses of C1, C2, PH, FYVE, FERM and other domains have led to an unprecedented amount of information on the molecular interactions of these signaling proteins with regulatory lipids. A wave of studies using GFP-tagged membrane binding domains as reporters has led to new quantitative insights into the kinetics of these signaling mechanisms.

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Abbreviations

C1	protein kinase C homology-1
C2	protein kinase C homology-2
cPKC	conventional (Ca^{2+} -dependent) protein kinase C
cPLA ₂	cytosolic phospholipase A ₂
DIG	detergent-insoluble glycoprotein-enriched domain
D-PS	phosphatidyl D-serine
ENTH	epsin amino-terminal homology
ER	endoplasmic reticulum
FERM	Four-point-one-ezrin-radixin-moesin
FYVE	Fab1-YOTF-Vac1-EEA1
GFP	green fluorescent protein
IP ₃	inositol (1,4,5)-trisphosphate
L-PS	phosphatidyl L-serine
nPKC	novel (Ca^{2+} -independent C2-domain containing) protein kinase C
PA	phosphatidic acid
PC	phosphatidylcholine
PH	pleckstrin homology
PKC	protein kinase C
PI3K	phosphoinositide 3-kinase
PIP ₂	phosphatidylinositol 3-phosphate
PIP ₃	phosphatidylinositol (4,5)-bisphosphate
PIP ₃	phosphatidylinositol (3,4,5)-trisphosphate
PIPK	phosphatidylinositol phosphate kinase
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
VHS	Vps27p, Hrs and STAM

Introduction

A great many signaling proteins redistribute within cells in response to receptor-stimulated lipid turnover. Many have been identified in the recent past and the number keeps increasing. These lipid-mediated protein-targeting mechanisms are not restricted to canonical signaling processes; they also play key roles in membrane trafficking and in anchoring

cytoskeletal structures. The action of these lipids in regulating protein localization depends on their binding to discrete protein domains [1]. In this review, we explore the intricate relationship between the structure of such domains and the lipid-mediated subcellular localization of signaling proteins.

The hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP₂) by phospholipase C (PLC) is the classic example of a receptor-activated pathway that generates a lipid second messenger signal, in this case diacylglycerol. The other hydrolysis product is the soluble second messenger inositol (1,4,5)-trisphosphate (IP₃), which stimulates the release of Ca^{2+} ions from intracellular stores. A small 5 kDa Zn^{2+} -containing domain, the protein kinase C homology-1 (C1) domain, has been identified in protein kinase C (PKC) and other signaling proteins as the locus responsible for diacylglycerol binding (Figure 1a,b; [2]). Much recent attention has centered on another lipid second messenger, phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), that can be produced by receptor-stimulated activation of phosphoinositide 3-kinase (PI3K). Certain proteins that contain pleckstrin homology (PH) domains translocate to the plasma membrane in response to PI3K activation [3–5], whereas others translocate in response to PIP₂ synthesis by phosphatidylinositol phosphate kinases (PIPKs) [4–6,7••].

It should be noted that several lipid-directed targeting mechanisms involve lipids whose concentration appears to be less acutely regulated. Although translocation may in these cases not occur as a result of changes in lipid concentration, new lipid-binding interactions may be driven instead by Ca^{2+} binding to the domain or by other regulatory events. The Ca^{2+} -mediated interaction of the protein kinase C homology-2 (C2) domain of cytosolic phospholipase A₂ (cPLA₂) with neutral lipids [8], and the interactions of C2 and C1 domains of PKC with phosphatidylserine (PS) [9] are two important examples that will be discussed below (Figure 1c).

The generally high specificity of lipid-binding domains for their lipid ligands has led to the idea that GFP conjugated with lipid-interaction domains can be used as fluorescent probes in living cells for measuring local lipid second messenger signals. This was first shown using a GFP–C1-domain to measure diacylglycerol signals [10], a GFP–PH-domain to measure phosphoinositide signals [11] and a GFP–C2-domain to measure Ca^{2+} signals [12]. Fluorescent translocation probes have recently been reviewed [4,13,14]. Here we evaluate the structural and cellular basis for membrane selectivity and the translocation of proteins with lipid-binding domains.

A perpetual search for short relationships

How do proteins that contain lipid-interaction domains find their newly created targets in a subcellular membrane?

A picture has emerged of a restless subclass of signaling proteins that spend a significant fraction of their time diffusing through the cytosol in search of binding partners. Depending on the fraction of protein that is available for diffusion and the type of binding interactions, the binding to new membrane sites can occur, in some cases, in less than one second (e.g. for conventional PKC [12]). The continued redistribution of many of these proteins is guaranteed by relatively low lipid–protein binding affinities, in the near micromolar range, that are readily reversible. This suggests that lipid second messenger binding interactions are well suited to rapidly localize signaling proteins to specific subcellular membranes, as the lipid composition can be selectively regulated within a particular membrane on a timescale of seconds to minutes.

The question of whether lipid second messengers are uniform targets within a membrane has been debated for many years and recent imaging of GFP-conjugated PH domains has shown that gradients and regionally localized PIP₂ and PIP₃ lipids indeed exist in the plasma membrane [15[•],16[•],17[•]]. Lipids may also be enriched in even smaller microdomains known as rafts or detergent-insoluble glycoprotein-enriched domains (DIGs; e.g. [18,19]) although the evidence for an actual spatial confinement of lipids to such structures is still debated. As different lines of experimental evidence suggest that the plasma membrane and internal membranes are fundamentally heterogeneous structures, organized by multiple interactions between plasma membrane proteins, cytoskeletal components and lipids themselves, the problem of identifying cellular functions for this lipid heterogeneity will remain a challenge for years to come. We will now focus on lipids as part of subcellular membranes without further discussing the interesting possibility of localized signaling functions within these membranes.

Diacylglycerol and Ca²⁺ signals trigger different recruitment mechanisms via C1 and C2 domains

The PLC/PKC pathway is a paradigm for regulated protein translocation induced by a cell-surface signal. The hydrolysis of PIP₂ by receptor-stimulated PLC isozymes leads to an increase in the intracellular Ca²⁺ concentration and increased diacylglycerol levels in the plasma membrane. Conventional (Ca²⁺-dependent) PKC (cPKC) and novel (Ca²⁺-independent C2-domain-containing) PKC (nPKC) isoforms interact with membranes through two conserved domains, C1 and C2, as well as via a basic pseudosubstrate region, which is near the amino terminus in cPKC (reviewed in [9]). Increased Ca²⁺ levels stimulate cPKC translocation by binding to its C2 domain and increasing its affinity for acidic phospholipids (Figure 1d). This initial translocation then facilitates the binding of diacylglycerol to the two C1 domains, which leads to tighter membrane binding and to the activation of the cPKC enzymes. Diacylglycerol promotes the binding of PKC to membranes by positioning itself into a groove in the hydrophobic tip of its C1 domain and anchoring it tightly to membranes

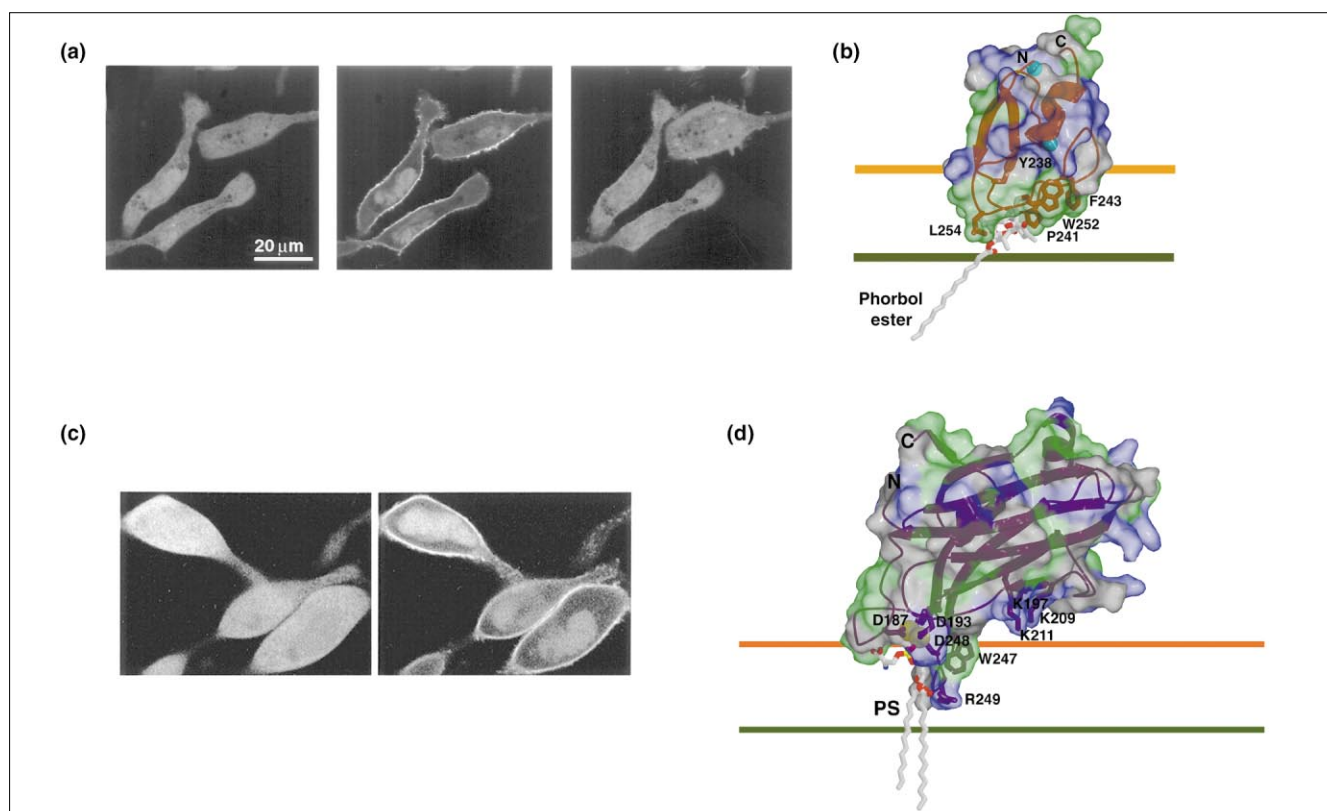
(Figure 1b). In nPKCs this diacylglycerol binding appears to be sufficient to induce the initial translocation. In all cPKC and nPKC isoforms activation of the kinase is thought to be closely coupled to its translocation and binding interactions with diacylglycerol and other lipids.

Although the C2 domain of cPKC isoforms localizes the enzyme, at least initially, to the plasma membrane, the C2 domain of cPLA₂ drives the enzyme to the endoplasmic reticulum (ER) and other internal membranes in response to an increase in Ca²⁺ concentration. In contrast to the PKC C2 domain, the one from cPLA₂ binds to neutral membranes, which are rich in phosphatidylcholine (PC), in preference to acidic ones [20,21]. Therefore, although it is the increased Ca²⁺ levels that provide the on-switch for translocation of cPLA₂, the intracellular localization appears to be driven to a large extent by the more neutral lipid composition of the cytosolic leaflet of the ER and possibly other internal membranes [8,21]. Many questions remain about just how important lipid composition is in directing proteins to bind to specific cellular membranes, in no small part because of limitations of the data on the lipid composition of various internal membranes.

It has long been known that PKC requires the acidic lipid phosphatidyl L-serine (L-PS) for enzyme activity. PS is abundant in the cytoplasmic leaflet of the plasma membrane. Because the Ca²⁺-bound C2 domain of PKC interacts with acidic phospholipids, it might have been assumed that this was the origin of the stereospecific requirement for L-PS. However, the C2 domain binds equally well to both the phosphatidyl D-serine (D-PS) and the L-PS isomers. Consistent with non-stereospecific binding, a recent crystal structure of the PKC α C2 domain complexed with a short chain L-PS suggests at least two different binding modes, arguing against a high level of stereospecificity [22]. Newton and co-workers [23[•]] have now shown that the origin of L-PS stereospecificity lies in the C1, not the C2, domain, although a different interpretation has also been advanced [24[•]]. The C1 domain structure lacks a well defined binding pocket for any lipid other than diacylglycerol. On the other hand, stereospecific recognition of lipids at a membrane surface requires only two direct contacts with the protein, as opposed to the usual three-point contact requirement that occurs away from the membrane. Two-point contact allows lipids other than diacylglycerol to bind as a less-specific interaction is required. The asymmetry of the interface provides the additional element of stereospecificity. This raises the interesting question of how PS and diacylglycerol interactions with the C1 domains orchestrate membrane targeting and kinase activation. The latest results do not provide the answer, but they set the stage for the experiments needed to answer the question.

Downstream of the PLC/PKC signaling system is a different type of lipid targeting mechanism that relies on the phospholipase D (PLD)-mediated production of phosphatidic

Figure 1



C1 and C2 domains. (a) Translocation of the GFP-tagged PKC-γ C1A domain. Timepoints before (left), 90s after (middle) and 300s after (right) activation of the IgE receptor, reproduced from [10]. (b) Membrane-docked structure of the C1B domain of PKC-δ complexed with phorbol 13-acetate (PDB entry 1ptr) modified from [1]. A myristate tail has been modeled onto the phorbol ester. The secondary structure and molecular surface of the domain is shown. Surfaces are colored according to the underlying residue type – hydrophobic (green) or basic (blue). Selected specific and nonspecific contact residues are shown. The domain is positioned so that known membrane-interacting residues penetrate the membrane and basic patches are proximal to the membrane surface. The membrane leaflet is divided into an interfacial zone and a hydrophobic

core (each ~15 Å thick) and is drawn to scale. The two bound Zn^{2+} ions are shown in cyan. (c) Translocation of the GFP-tagged C2 domain of PKC-γ immediately before (left) and after (right) ionomycin addition, reproduced from [12] with permission. (d) Membrane-docked model of the structure of PKC-α bound to PS, colored as in (b). The two bound Ca^{2+} ions are shown in yellow. The dibutyl moiety in the crystal structure (PDB entry 1dsy, [22]) has been replaced by a dimyristoyl moiety and the orientation of the acyl groups have been modified such that they point into the hydrocarbon core of the bilayer. The binding of PS to the C2 domain in this complex is non-stereospecific [23] and probably represents a general mode of Ca^{2+} -dependent binding to anionic phospholipids.

acid (PA). Recent studies showed that a PA-mediated translocation of Raf to the plasma and endosomal membranes is mediated by a 4 kDa region of Raf (amino acids 390–426; [25,26]). Similar to the Ca^{2+} -triggered translocation of cPKCs, which only minimally activates PKC, the translocation of Raf does not appear to activate kinase activity but, instead, facilitates its activation by GTP-Ras. As for two other proposed lipid mediators, ceramide and sphingosine-1 phosphate, it will now be important to directly show whether specific domain structures exist that selectively bind these novel lipid second messengers.

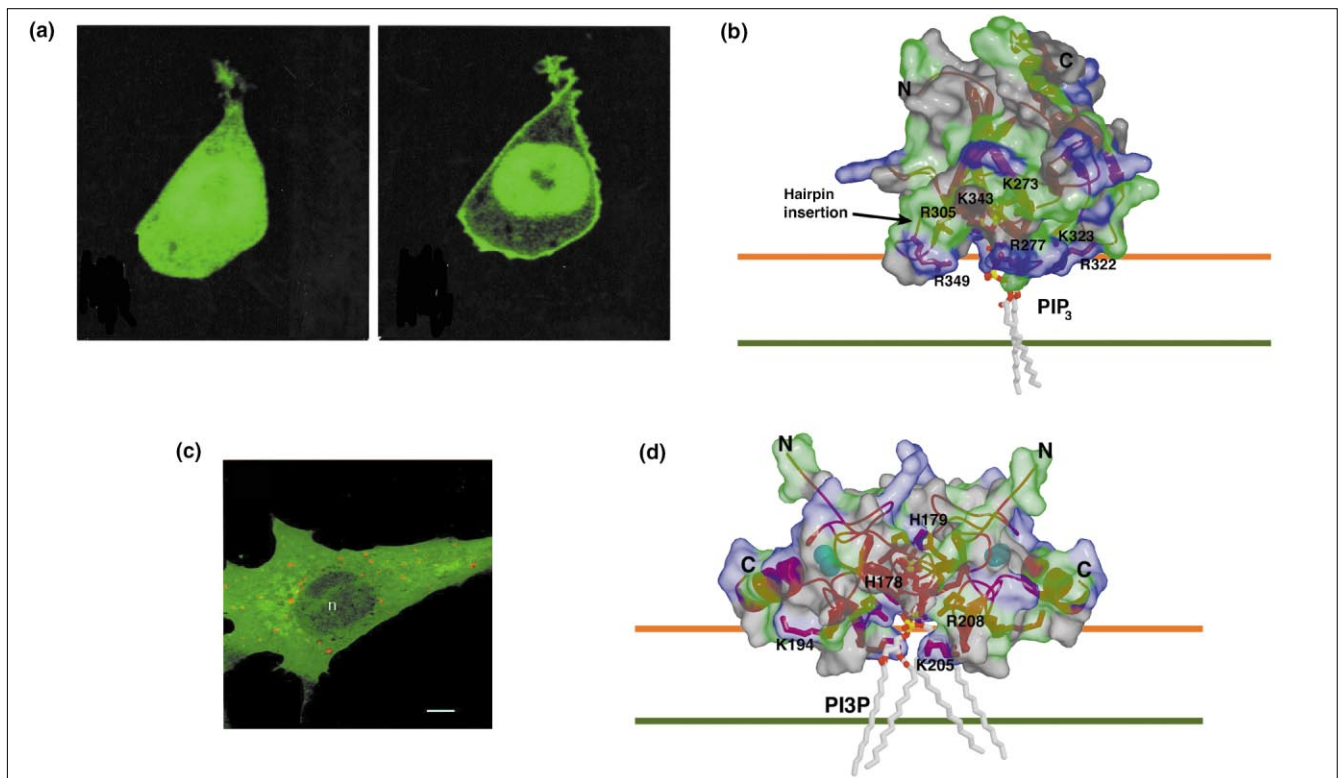
3-phosphoinositide recognition: many ways to skin a cat

It is now clear that the phosphorylation of the D-3 position of phosphoinositides plays a central role in signaling, membrane trafficking and an array of other key processes

in many aspects of eukaryotic cell biology. Many mammalian cell surface receptors signal through class I PI3Ks, which can phosphorylate PIP_2 and PIP_3 . PIP_2 and PIP_3 transmit signals by virtue of their specific interactions with PH-domain-containing proteins. The large majority of PH domains do not specifically bind 3-phosphoinositides [6,27] but those that do have recently been under the spotlight. These include the PH domains of the kinases Akt, PDK1 and Btk, and the Arf GTP exchange factors ARNO and Grp1 (Figure 2a; [28]).

Early ideas about how PIP_3 might bind to the PH domains were derived from the structure of the PIP_2 headgroup bound to the PH domain of PLC-δ. However, the first structure of a PIP_3 headgroup bound to the PH domain from Btk [29] revealed a surprise: the inositol ring was flipped nearly 180° about the axis between the

Figure 2



PH and FYVE domains. (a) Translocation of the GFP-tagged Grp1 PH domain before (left) and after (right) PI3K activation, reproduced from [28] with permission. (b) Membrane-docked structure of the Grp1 PH domain complex with PIP_3 (PDB entry 1fyg, [30*]), colored as for Figure 1b. A dimyristoyl group has been modeled. (c) Localization of GFP-tagged tandem FYVE domains constructed as a probe for PI3P , reproduced from [34*] with permission. The subcellular distribution of

GFP-(FYVE)₂ (green) is compared with a late endosome marker, LBPA (red). Scale bar, 5 μm . (d) Membrane-docked structure of the dimeric FYVE domain of Hrs (PDB entry 1dvp, [35*]). The PI3P headgroups are modeled on the basis of bound citrate molecules and on the PI3P site previously located by mutagenesis [33] and analysis of crystal contacts [32], colored as for Figure 1b.

1 and 4 positions, leading the 3-phosphate and 5-phosphate of PIP_3 to exchange positions as compared with the 3-hydroxyl and 5-phosphate of PIP_2 bound to PLC- δ . The Btk PH domain contains a large basic insertion in its $\beta 1$ - $\beta 2$ loop that forms extensive interactions with the 5-phosphate.

Other 3-phosphoinositide-specific PH domains, such as that of Grp1, lack many of the basic residues found in Btk. Two new structures of the Grp1 PH domain complexed with the PIP_3 headgroup (Figure 2b; [30*,31*]), as well as a structure of the DAPP1/PHISH PH domain [31*], clear up this mystery. The Grp1 PH domain contains two additional β strands inserted between $\beta 6$ and $\beta 7$ of the core PH-domain fold. Basic residues on this β hairpin interact with the 3-phosphate. Thus, at least two major subsets of PH domains specifically recognize PIP_3 using residues from different parts of the structure. The structural differences are manifested in sequence motifs that have now been used to identify novel PIP_3 receptors [30*,31*]. These results strengthen the view that PH-domain structures are markedly divergent, even among domains with similar functions.

When it comes to binding 3-phosphoinositides, there are even more ways to skin the cat. Phosphatidylinositol 3-phosphate (PI3P), a lipid that is more important for endosomal traffic direction than for receptor-linked signaling, interacts not with PH domains, but rather with the small Zn^{2+} -containing Fab1-YOTP-Vac1-EEAI (FYVE) domain. At barely half the size of a PH domain, the FYVE domain uses an elegant economy of interactions to specifically bind its target, as revealed by the structure of the monomeric FYVE domain of Vps27p [32]. It was already clear from early work that the PI3P affinity for the monomeric FYVE domain was inadequate for endosomal targeting [33]. Additional regions, such as the Rab5 interaction domain of EEA1 [33] or tandem FYVE domains (Figure 2c; [34*]), were required to enhance the affinity of the binding interaction. The structure of a dimeric FYVE domain from Hrs [35*] suggested that the dimer interface itself was directly involved in ligand recognition (Figure 2d). The close proximity of the two PI3P -binding sites in the Hrs FYVE structure is consistent with this view. The picture should become clearer in the future as more direct evidence about the oligomeric state of membrane-bound FYVE domains is obtained, together with a much-needed crystal structure of a FYVE domain bound to PI3P .

Phosphatidylinositol (4,5)-bisphosphate: 'I am my own second messenger'

PIP₂ has come into its own as a second messenger, notably in regulation of the cytoskeleton (e.g. [7•]), plasma membrane channels and transporters (e.g. [36]), as well as in membrane trafficking [37]. The functional dissection of the roles played by this key lipid second messenger is made difficult by the large number of binding partners, which includes proteins with PH domains, Four-point-one-ezrin-radixin-moesin (FERM) domains and probably many other partners, such as channels and transporters with structural features that are not yet known. Nevertheless, the past years have led to a rapid advancement of our understanding of structural and functional aspects of this important lipid mediator of cell function.

The localization of the PIPKs responsible for the regulated synthesis of PIP₂ is, itself, highly regulated. The PIPKs contain an active site loop that corresponds structurally to the activation loop of the protein kinases. This 20-amino-acid loop is responsible for nearly all of the phosphoinositide specificity observed *in vitro* [38•]. More surprisingly, this loop appears to have a major role in dictating the subcellular localization of PIPKs. Chimeric PIPKs were made in which the activation loop of type II β PIPK was inserted in type I β PIPK and vice-versa. These chimeras localize as expected for wild-type enzymes with the same activation loop sequence. This suggests that the subcellular distribution of the PIPK substrates phosphatidylinositol 4-phosphate and phosphatidylinositol 5-phosphate could be influencing PIPK localization. It is equally possible that the activation loops contain distinct but overlapping determinants for substrate specificity and localization that operate by different mechanisms.

The FERM-domain-containing proteins ezrin, radixin and moesin have emerged as important players in regulating the cytoskeleton by linking actin filaments to adhesion proteins. The activity of these proteins is regulated by PIP₂, although the precise mechanism of activation is under debate. The first structure of a FERM-domain-containing protein, that of moesin [39•], revealed a PH-domain fold as a substructure within the FERM and a putative PIP₂-binding site. The moesin structure also revealed a carboxy-terminal domain that masks the protein-protein interaction site. Now the structure of an IP₃ complexed with another FERM domain, that of radixin [40•], has been solved. The IP₃ binds to a cleft formed by the PH-domain-like substructure and a ubiquitin-fold substructure. This site does not correspond to sites on PH domains that bind phosphoinositides. There are relatively few interactions compared with the higher affinity PH-domain-phosphoinositide complexes, and the interactions present are mainly with the 4-phosphate. A small conformational change appears to occur on IP₃ binding, but it is not clear whether it is extensive enough to support an allosteric mechanism of activation as opposed to a simple targeting mechanism.

New targeting mechanisms from genomics and proteomics

Genomics and proteomics are bringing potential membrane-targeting domains into view faster than new mechanisms can be characterized. The epsin amino-terminal homology (ENTH) domain [41] and Vps27p, Hrs and STAM (VHS) domain [42] provide two such examples. The domains were discovered by sequence analysis and have received considerable attention, despite their lack of a well established function. These domains are found at the amino termini of many key proteins involved in the regulation of endocytosis. Although classified separately on the basis of sequence homology, the newly solved three-dimensional structures of the VHS domains of Hrs and Tom1 [35•,43•] and the ENTH domain of epsin-1 [44•] show they are nearly identical eight-helical bundles, differing only in the conformation of the last helix. Despite the lack of sequence identity among these domains, they all have in common a conserved basic face. The face is relatively flat, lacking a distinct pocket. This structure is typical of membrane-binding domains that bind to acidic phospholipids with low-to-moderate specificity and affinity. The VHS domain of EAST appears to be essential for its localization to the plasma membrane [45] and the VHS domains of the GGA proteins diffusely localize to several intracellular membranes [46]. It remains to be seen whether ENTH-domain-mediated and VHS-domain-mediated localization represents an important new class of lipid-based membrane-targeting mechanisms or whether protein-protein interactions, such as those documented for the ENTH domain [44•,47], are more important.

Conclusions

Why have cells chosen lipids in addition to proteins for targeting to subcellular membranes? Lipids can do a few things in targeting that proteins can't. First, relatively large numbers of lipid-binding partners can exist in cells, which makes it possible to target a large number of different proteins to particular membranes without saturating the binding sites. Second, lipid-binding partners can readily be produced and degraded by enzymatic activities, which enables changes in the distribution of entire families of signaling proteins on the timescale of seconds to minutes. Third, lipid-binding interactions are often low affinity and reversible, which enables rapid protein equilibration across the cell if relatively more lipid is produced at a different site. Fourth, lipids are small compared with proteins, so that several lipids or combinations of lipids and proteins can be used to target proteins to distinct subcellular membranes. This may allow for selective targeting to specific membranes even though the core of the lipid-interaction domain can be identical.

How far have we come and what is left to do? Structural analysis of lipid interaction domains has led to a more precise picture of the action of many lipid second messengers, notably diacylglycerol, PIP₃, PIP₂ and PIP. On the other hand, the mechanisms of action of other signaling lipids,

such as PA and ceramide, are not yet clear. More lipid-based targeting mechanisms will certainly become known over the next few years, but genomic and proteomic analysis of signaling domains as a class is likely to place a cap on their number. The identification of the remaining lipid-targeting domains and their structural and mechanistic analysis continues.

It will also be critically important to experimentally address the question of membrane-binding selectivity, as much evidence suggests that membrane-binding interactions involve multiple lipid and possibly protein components in addition to the main lipid partner. This raises the important point that we actually know very little about the lipid composition of different subcellular membranes as the biochemical measurements are made difficult by the rapid turnover of the most interesting lipids, and this cannot cleanly be prevented during membrane fractionation.

In addition to identifying all lipid-binding domains and all lipid-mediated subcellular localization mechanisms, it will also be important to begin to break down how these targeting mechanisms organize subcellular signaling events in space and time. Many of the studies on the organization of cellular signaling networks will profit from the now available methodology that allows visualization of two or more membrane-targeted signaling proteins in individual cells using different fluorescent protein tags.

Acknowledgements

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